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TITLE: NOVEL ENONE REDUCTASES, METHODS FOR PRODUCING SAME, AND METHODS FOR SELECTIVELY REDUCING A CARBON-CARBON DOUBLE BOND OF AN α,β-UNSATURATED KETONE USING THE REDUCTASES

APPLICANT: HIROAKI YAMAMOTO AND NORIHIRO KIMOTO

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NOVEL ENONE REDUCTASES, METHODS FOR PRODUCING SAME, AND METHODS FOR SELECTIVELY REDUCING A CARBON-CARBON DOUBLE BOND OF AN α,β-UNSATURATED KETONE USING THE REDUCTASES

TECHNICAL FIELD

The present invention relates to novel enone reductases which are useful for reducing an α,β-unsaturated bond of an α,β-unsaturated ketone (enone), and polynucleotides encoding such reductases, as well as methods for producing the reductases and methods for selectively reducing a carbon-carbon double bond of an α,β-unsaturated ketone using the reductases or a polypeptide having homology with the enzyme.

BACKGROUND

Ketones are compounds that are widely used as raw materials in the synthesis of organic compounds. In addition, ketones are also important raw materials for the production of optically active alcohols and optically active amines that are optically active intermediates important in the synthesis of pharmaceuticals. For example, α,β-unsaturated ketones obtainable by the condensation reaction of aldehydes and ketones are useful as precursors for these ketones.

For example, 3-methyl-3-penten-2-one can be readily prepared by the condensation of acetaldehyde and 2-butane (J. Amer. Chem. Soc., 81:1117-1119, 1959).

Various ketones can be obtained by selectively reducing the α,β-unsaturated bonds of α,β-unsaturated carbonyl compounds. Hydrogenation reactions using Ni catalyst or Pd-C catalyst ("Catalytic Hydrogenation Reaction" p135, Tokyo Kagaku Dojin (1987)) are methods known in the art for selectively reducing the α,β-unsaturated bonds alone, without reducing any carbonyl groups. However, these methods have the following problems to be solved: (1) carbonyl groups may be also reduced by continuing the reaction; (2) metals, which have adverse effects on the environment, are used as the catalysts; and (3) high-pressure hydrogen gas is required for the reaction. Importantly, the reduction of carbonyl groups leads to decrease of the ketone yield.

On the other hand, methods using organisms as follows are reported as methods for selectively reducing carbon-carbon double bonds of α,β-unsaturated ketones using biological reactions:

- plant cells (J. Nat. Prod. 56:1406-1409, 1993);
- baker's yeast (Tetrahedron Lett. 52:5197-5200, 1978; Bull. Chem. Soc. Jpn. 64:3473-3475, 1991; Tetrahedron Asym. 6:2143-2144, 1995; etc.); and
- fungus (J. Org. Chem. 47:792-798, 1982).

However, these biological methods have their own problems such as: (1) carbonyl groups are also reduced; (2) low reactivity; and (3) cell preparation on a large-scale is difficult. Further, various types of enone reductases derived from these organisms have been reported. However, genes encoding these reductases remain to be cloned, and it is therefore hard to conveniently prepare these enzymes on a large scale.

In addition to the above-mentioned reductases of the α,β-unsaturated carbonyl compounds, such reductases as follows have been reported. These reductases are not suitable for industrial applications because either the substrate specificity of these reductases remains to be clarified or the selectivity for the α,β-unsaturated bond is low.

- *Clostridium tyrobutyricum*-derived 2-enoate reductase (E.C.1.3.1.31) (J. Biotechnol. 6:13-29, 1987);
- *Clostridium kluyveri*-derived acryloyl-CoA reductase (Biol. Chem. Hoppe-Seyler 366:953-961, 1985);
- Enone reductase YER-2 purified from baker's yeast (Kawai et al. ((Kyoto University), The 4th Biocatalyst symposium, Abstract p58 (2001));
- Enone reductases purified from a baker's yeast EI and EII (Eur. J. Biochem. 255:271-278, 1998);
- Enone reductase (verbenone reductase; also referred to as p90) derived from tobacco (*Nicotiana tabacum*) cells (J. Chem. Soc., Chem. Commun. 1426-1427, 1993; Chem. Lett. 850-851, 2000);
- Carvone reductase (also referred to as enone reductase-I), which is an enone reductase derived from tobacco (*Nicotiana tabacum*) cells (Phytochemistry 31:2599-2603, 1992):
- Enone reductase-II, p44, and p74, which are enone reductases derived from tobacco (*Nicotiana tabacum*) cells;
- Enone reductases purified from *Euglena gracilis* and *Astasia longa*, which are plant species (Phytochemistry 49, 49-53 (1998)); and
- Enone reductase purified from rat liver (Arch. Biochem. Biophys. 282:183-187, 1990).

SUMMARY

The object of the present invention is to provide novel enone reductases, which have an enzyme activity to selectively reduce the α,β-unsaturated bonds of α,β-unsaturated ketones to produce α,β-saturated ketones, and genes encoding the reductases. Another object of the present invention is to provide methods for selectively reducing the carbon-carbon double bonds of α,β-unsaturated ketones using the reductases and organisms producing the reductases.

The present inventors screened enzymes producing 2-butanone from methyl vinyl ketone and found that *Kluyveromyces lactis* has the activity of interest. Then, they purified the enzyme having the activity of interest from fungal cells of *Kluyveromyces lactis*, and revealed the properties thereof. They confirmed that the enzyme selectively reduced the α,β-unsaturated bonds of α,β-unsaturated ketones in a β-nicotinamide adenine dinucleotide phosphate (NADPH)-dependent manner, and that the enzyme has substantially no activity to reduce ketones. Further, the present inventors cloned a gene encoding the enzyme, clarified the structure thereof, and verified that the gene was novel. In addition, they overexpressed the gene in a heterologous organism to obtain a transformed strain having higher selectivity and higher activity at the same time to reduce the α,β-unsaturated bonds of α,β-unsaturated ketones in a NADPH-dependent manner. Furthermore, they found that selective reduction of the carbon-carbon double bonds of α,β-unsaturated ketones can be achieved by the enzyme, homologues thereof, cells producing them, and so on, and thus, completed the present invention. Hereinafter, β-nicotinamide adenine dinucleotide phosphate is referred to as NADP; β-nicotinamide adenine dinucleotide as NAD; and the reduced forms thereof as NADPH and NADH, respectively.

More specifically, the present invention relates to the following enone reductases, polynucleotides encoding the reductases, methods for producing the reductases, and methods for selectively reducing carbon-carbon double bonds of α,β-unsaturated ketones using the reductases or polypeptides having homology to such reductases.

[1] An enone reductase having the following physicochemical properties:

(A) Action:

The enzyme reduces the carbon-carbon double bonds of the α,β-unsaturated ketones, using NADPH as an electron donor, to produce the corresponding saturated hydrocarbon;

(B) Substrate specificity:

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

(1) the enzyme reduces the carbon-carbon double bonds of the α,β-unsaturated ketones but has substantially no activity to reduce ketones;

(2) the enzyme exhibits a significantly higher activity with NADPH than with NADH as the electron donor;

(3) the enzyme does not substantially act on substrates, wherein both substituents at the β carbon relative to the ketone are not hydrogen; and

(4) the enzyme does not substantially act on substrates, wherein the carbon-carbon double bond is present in the cyclic structure; and

(C) Optimal pH:

pH 6.5-7.0;

[2] The enone reductase of [1], wherein the reductase further has the following physicochemical properties:

(D) Optimum temperature:

37-45°C

(E) Molecular weight:

The molecular weight of the reductase determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by gel filtration is about 43,000 and about 42,000, respectively;

[3] The novel enone reductase of [1], which is derived from the genus *Kluyveromyces*;

[4] A method for obtaining the enone reductase of [1], comprising the step of culturing a microorganism belonging to the genus *Kluyveromyces* and having the ability of producing to the novel enone reductase of [1];

[5] The method of [4], wherein the microorganism belonging to the genus *Kluyveromyces* is *Kluyveromyces lactis*;

[6] A polynucleotide encoding a polypeptide having enone-reducing activity selected from the group of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1;

(b) a polynucleotide encoding the amino acid sequence of SEQ ID NO:2;

(c) a polynucleotide encoding a polypeptide comprising the the amino acid sequence of SEQ ID NO:2, in which one or more amino acids are substituted, deleted, inserted, and/or added;

(d) a polynucleotide hybridizing under stringent conditions with a polynucleotide consisting of the nucleotide sequence of SEQ ID NO:1; and

(e) a polynucleotide encoding an amino acid sequence exhibiting 60% or higher percent identity to the amino acid sequence of SEQ ID NO:2;

[7] A polypeptide encoded by the polynucleotide of [6];

[8] A recombinant vector comprising the polynucleotide of [6];

[9] The recombinant vector of [8], wherein a polynucleotide encoding a dehydrogenase catalyzing oxidation-reduction reactions using NADP as a coenzyme is further inserted;

[10] A transformant harboring the polynucleotide of [6] or the vector of [8] in an expressible manner;

[11] A method for producing the polypeptide of [7], comprising the step of culturing the transformant of [10];

[12] A polynucleotide encoding a polypeptide having enone-reducing activity selected from the group of:

(a) a polynucleotide comprising the nucleotide sequence of any one of SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7;

(b) a polynucleotide encoding a polypeptide comprising the amino acid sequence of any one of SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8.

(c) a polynucleotide encoding the amino acid sequence comprising the sequence of any one of SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8, in which one or more amino acids are substituted, deleted, inserted and/or added;

(d) a polynucleotide hybridizing under stringent conditions with a polynucleotide consisting of the nucleotide sequence of any one of SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7; and

(e) a polynucleotide encoding an amino acid sequence exhibiting 60% or higher percent identity to the amino acid sequence of any one of SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8;

[13] A polypeptide encoded by the polynucleotide of [12];

[14] A recombinant vector wherein the polynucleotide of [12] has been inserted;

[15] The recombinant vector of [14], wherein a polynucleotide encoding a dehydrogenase catalyzing oxidation-reduction reactions using NADP as a coenzyme is further inserted;

[16] A transformant harboring the polynucleotide of [12] or the vector of [14] in an expressible manner;

[17] A method for producing the polypeptide of [13], comprising the step of culturing the transformant of [16];

[18] A method for selectively reducing the carbon-carbon double bonds of α,β-unsaturated ketones comprising the step of reacting the α,β-unsaturated ketone with enzyme active materials selected from the group of: (1) enone reductase of [1]; (2) the polypeptide of [7]; (3) the polypeptide of [13]; (4) a microorganism producing the enzyme or polypeptide; and (5) processed products of the microorganism; and

[19] The method of [18], wherein the microorganism producing the enzyme or polypeptide is the transformant of [10] and/or [16].

DESCRIPTION OF DRAWINGS

FIG. 1 is a photograph showing the electrophoretic pattern of SDS-PAGE. Lane 1 represents the molecular weight marker; lane 2 the enzyme obtained in Example 1.

FIG. 2 shows the pH dependency of the methyl vinyl ketone-reducing activity of the enzyme obtained in Example 1.

FIG. 3 shows the temperature dependency of the methyl vinyl ketone-reducing activity of the enzyme obtained in Example 1.

FIG. 4 is a schematic illustration of plasmid pSE-KLR1 containing the enone reductase gene.

DETAILED DESCRIPTION

The present invention provides enzymes having the following physicochemical properties:

(A) Action:

The enzyme reduces the carbon-carbon double bonds of α,β-unsaturated ketones using NADPH as an electron donor to produce a corresponding saturated hydrocarbon.

(B) Substrate specificity:

(1) the enzyme reduces the carbon-carbon double bonds of α,β-unsaturated ketones but does not substantially have the activity to reduce ketones;

(2) the enzyme exhibits a significantly higher activity with NADPH than with NADH as the electron donor;

(3) the enzyme does not substantially act on substrates wherein both substituents at the β carbon relative to the ketone are not hydrogen; and

(4) the enzyme does not substantially act on substrates wherein the carbon-carbon double bonds are present in the cyclic structure.

(C) Optimal pH:

pH 6.5-7.0.

Preferably, the enone reductase of the present invention further has the following physicochemical properties:

(D) Optimal temperature:

37-45°C;

(E) Molecular weight:

The molecular weight of the reductase determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (hereinafter abbreviated as SDS-PAGE) and by gel filtration is about 43,000 Da and about 42,000 Da, respectively.

"The enzyme exhibits a significantly higher activity with NADPH than with NADH" means that the reactivity is at least twice or higher, preferably 3 times or higher, more preferably 5 times or higher. The difference in the relative reactivities to NADPH and NADH can be compared using methods such as those shown in the Examples. Specifically, ketones are generated in the presence of either of these electron donors using the same type of α,β-unsaturated ketone as the substrate. The comparison of reactivity can be conducted by comparing the amounts of consumed NADPH and NADH, respectively.

Further, as used herein, "enone reductase substantially does not have the activity to reduce ketones" or "enone reductase substantially does not act on the substrate" specifically means that the activity is 1% or less of the activity of the reductase to reduce an olefin of a methyl vinyl ketone.

The enzyme of the present invention can be purified from microorganisms producing the enzyme by a standard protein purification method. For example, the enzyme can be purified by lysing the fungal cells, carrying out protamine-sulfate precipitation and centrifugation, salting out the centrifugal supernatant with ammonium sulfate, and then isolating by the combined use of anion exchange chromatography, hydrophobic chromatography, affinity chromatography, gel filtration, etc.

According to the present invention, the enone-reducing activity can be verified as follows. As used herein, "enone" refers to α,β-unsaturated ketones. An exemplary assay for measuring the enone-reducing activity is as follows:

A reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 0.2 mM NADPH, 20 mM methyl vinyl ketone, and the enzyme is reacted at 30°C, and the decrease in absorbance at 340 nm, which is associated with the decrease of the amount of NADPH, is measured. 1 U is defined as the amount of enzyme catalyzing a decrease of 1 μmol NADPH in one minute. Quantitative analysis of the polypeptide is conducted by pigment binding methods using the protein assay kit (Bio-Rad Laboratories Inc.).

Enone reductases having physicochemical properties such as those described above can be purified, for example, from cultures of yeast belonging to the genus *Kluyveromyces*. *Kluyveromyces lactis*, among yeasts belonging to the genus *Kluyveromyces*, is particularly excellent in the production of enone reductase of the present invention. *Kluyveromyces lactis*, for example, IFO 0433, IFO 1012, IFO 1267,IFO 1673, and IFO 1903, can be used to

obtain the enone reductase of the present invention, which are available from the Institute for Fermentation, Osaka (IFO).

The above-mentioned microorganisms can be cultured in a medium, such as YM medium, that is generally used for the cultivation of fungi. After well grown, the fungal cells are harvested and lysed in a buffer containing reducing agents, such as 2-mercaptoethanol, and protease inhibitors, such as phenylmethane sulfonylfluoride, to give a cell-free extract. The enzyme can be purified from the cell-free extract by appropriate combinations of fractionation, based on the protein solubility (precipitation with organic solvents, salting out with ammonium sulfate, etc.); and by chromatographies such as cation-exchange, anion-exchange, gel filtration, hydrophobic, and affinity chromatography using chelate, dye, antibody, and so on. The enzyme can be purified as an electrophoretically homogeneous polypeptide, for example, by hydrophobic chromatography using phenyl-Sepharose, anion-exchange chromatography using MonoQ, hydrophobic chromatography using phenyl-Superose, and such.

The enone reductases of the present invention, that can be purified from *Kluyveromyces lactis,* should have the physicochemical properties described above as (A)-(C) and (D)-(E). The enone reductase of the present invention, that can be purified from *Kluyveromyces lactis,* is undoubtedly a novel enzyme which is different from α,β-unsaturated carbonyl-compound reductases known in the art.

For example, *Clostridium tyrobutyricum*-derived 2-enoate reductase (E.C.1.3.1.31) is known as a reductase of α,β-unsaturated carbonyl-compounds. This enzyme reduces (E)-2-methyl-2-butenoic acid in the presence of NADH and produces (R)-2-methylbutyric acid (J. Biotechnol. 6:13-29, 1987). Further, the enzyme acts on substrates, wherein the carbonyl group is contained as carboxylic acid, aldehyde, and keto acid; no activity of acting on ketones has been reported. Furthermore, the molecular weight of this enzyme is 800,000 to 940,000 Da as determined by gel filtration, and thus, is clearly different from the enzyme of the present invention, having a molecular weight is 43,000 Da determined by SDS-PAGE and 42,000 Da by gel filtration.

It has also been reported that a *Clostridium kluyveri*-derived acryloyl-CoA reductase has an ethyl vinyl ketone reductase activity (Biol. Chem. Hoppe-Seyler 366:953-961, 1985). This enzyme uses a reduced type of methyl viologen as the coenzyme and its molecular

weight has been determined to be 28,400 Da by gel filtration and 14,200 Da by SDS-PAGE. Therefore, this enzyme is quite different from the enzyme of the present invention.

In addition, a number of enone reductases purified from baker's yeast are reported. Kawai *et al.* at Kyoto University have purified an enone reductase (YER-2) from baker's yeast, and reported the enzymological characteristics thereof (The 4th Biocatalyst symposium, Abstract p58 (2001)). The optimal pH of YER-2 for the reaction is pH 7.5, and this indicates that this enzyme is quite different from the enzyme of the present invention (which has an optimal pH of pH 6.5-7.0). Wanner *et al.* reported the purification and characterization of two types of enone reductases (EI and EII) derived from the same baker's yeast (Eur. J. Biochem. 255:271-278, 1998). EII uses NADH as the coenzyme; and EI is a heterodimer having a molecular weight of 75,000, consisting of two subunits, 34,000 Da and 37,000 Da, as determined by SDS-PAGE. For similar reasons to those above, these enzymes are different from the enzyme of the present invention.

Many enone reductases (verbenone reductase (also referred to as p90), carvone reductase (also referred to as enone reductase-I, enone reductase-II, p44, p74) have been purified from cells of a plant species, tobacco (*Nicotiana tabacum*),and their characteristics have been reported. The verbenone reductase (p90) and p44 have activities to reduce cyclic α,β-unsaturated ketones (J. Chem. Soc., Chem. Commun. 1426-1427, 1993; Chem. Lett. 850-851, 2000), and thus, are different from the enzyme of the present invention. The carvone reductase uses NADH as the coenzyme (Phytochemistry 31:2599-2603, 1992); enone reductase-II can act on compounds, wherein no hydrogen atom exists at the β carbon of the α,β-unsaturated ketone ((R)-pulegone), as the substrate (Phytochemistry 31:2599-2603, 1992); and p74 has a molecular weight of 74,000 Da. Thus, all of these enzymes are quite different from the enzyme of the present invention.

In addition, enone reductases have been also purified from *Euglena gracilis* and *Astasia longa*, which are a kind of plant species (Phytochemistry 49:49-53, 1998). Both of these enzymes use NADH as the coenzyme, and thus are different from the enzyme of the present invention.

Further, with respect to animal species, an enone reductase has been purified from the liver of rat (Arch. Biochem. Biophys. 282:183-187, 1990). This enzyme is a monomeric enzyme with a molecular weight of 39,500. However, the reactivity to cyclic substrates, the

reactivity to substrates disubstituted at the β position, the optimal pH, and such properties have not yet been reported.

The present invention relates to isolated polynucleotides encoding an enone reductase and homologues thereof.

As used herein, an "isolated polynucleotide" is a polynucleotide the structure of which is not identical to that of any naturally occurring polynucleotide or to that of any fragment of a naturally occurring genomic polynucleotide spanning more than three separate genes. The term therefore includes, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule in the genome of the organism in which it naturally occurs; (b) a polynucleotide incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion polypeptide. Specifically excluded from this definition are polynucleotides of DNA molecules present in mixtures of different (i) DNA molecules, (ii) transfected cells, or (iii) cell clones; e.g., as these occur in a DNA library such as a cDNA or genomic DNA library.

Accordingly, in one aspect, the invention provides an isolated polynucleotide that encodes a polypeptide described herein or a fragment thereof. Preferably, the isolated polypeptide includes a nucleotide sequence that is at least 60% identical to the nucleotide sequence shown in SEQ ID NO:1. More preferably, the isolated nucleic acid molecule is at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, identical to the nucleotide sequence shown in SEQ ID NO:1. In the case of an isolated polynucleotide which is longer than or equivalent in length to the reference sequence, e.g., SEQ ID NO:1, the comparison is made with the full length of the reference sequence. Where the isolated polynucleotide is shorter than the reference sequence, e.g., shorter than SEQ ID NO:1, the comparison is made to segment of the reference sequence of the same length (excluding any loop required by the homology calculation).

Herein, the polynucleotides may be composed of naturally occurring polynucleotides, such as DNA and RNA, or they may contain artificially synthesized nucleotide derivatives.

There is no restriction on length of the polynucleotide of the present invention, but it preferably comprises at least 15 nucleotides.

A polynucleotide encoding an enone reductase of the present invention comprises, for example, the nucleotide sequence of SEQ ID NO:1. The nucleotide sequence of SEQ ID NO:1 encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2. This polypeptide, comprising the amino acid sequence of SEQ ID NO:2, is a preferred embodiment of the enone reductase of the present invention. Furthermore, the polynucleotide of the present invention includes those nucleotide sequences which encode the amino acid sequence of SEQ ID NO:2. There are 1 to 6 kinds of codons corresponding to an amino acid, and thus, a polynucleotide encoding the polypeptide consisting of the amino acid sequence of SEQ ID NO:2 is not restricted to the polynucleotide of SEQ ID NO:1, and there are multiple types of polynucleotides that are equivalent to the polynucleotide of SEQ ID NO:1.

The polynucleotides of the present invention include polynucleotides that have the amino acid sequence of SEQ ID NO:2 in which one or more amino acids are deleted, substituted, inserted and/or added yet which encode a protein having the enzyme activity of an enone reductase. For example, those skilled in the art can introduce substitution, deletion, insertion, and/or addition mutations into the polynucleotide of SEQ ID NO:1 by site-directed mutagenesis (Nucleic Acid Res. 10:6487, 1982; Methods in Enzymol. 100:448, 1983; Molecular Cloning 2nd Ed., Cold Spring Harbor Laboratory Press, 1989; PCR A Practical Approach, IRL Press pp. 200, 1991), and such.

Further, the polynucleotides of the present invention include polynucleotides that hybridize under stringent conditions to the polynucleotide consisting of the nucleotide sequence of SEQ ID NO:1, yet which encode a polypeptide having the enzyme activity of an enone reductase. The phrase "polynucleotides hybridizing under stringent conditions" refers to polynucleotides hybridizing using, for example, ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech) under conditions described in the manual (wash: 42°C, primary wash buffer containing 0.5x SSC) and using polynucleotide(s) selected from one or more sequences containing at least consecutive 20, preferably at least consecutive 30, for example, consecutive 40, 60 or 100 residues arbitrarily selected from the sequence of SEQ ID NO:1 as a probe polynucleotide. Also included in the invention is a

polynucleotide that hybridizes under high stringency conditions to the nucleotide sequence of SEQ ID NO:1 or a segment thereof as described herein. "High stringency conditions" refers to hybridization in 6x SSC at about 45°C, followed by one or more washes in 0.2x SSC, 0.1% SDS at 65°C.

Polynucleotides hybridizing under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO:1 include polynucleotides comprising a nucleotide sequence homologous to that of SEQ ID NO:1. It is highly probable that such polynucleotides encode polypeptides functionally equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO:2. Thus, based on the description herein, those skilled in the art can select polynucleotides encoding polypeptides having the enone reductase activity from such polynucleotides.

Further, the polynucleotides of the present invention include polynucleotides that have a percent identity of at least 60%, more preferably at least 70% or 80%, and further more preferably more than 90% to the polynucleotide encoding a polypeptide having the amino acid sequence of SEQ ID NO:2. As used herein, "percent identity" of two amino acid sequences or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990) modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Homology search of protein can readily be performed, for example, in DNA Databank of JAPAN (DDBJ), by using the FASTA program, BLAST program, etc. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3. Where gaps exist between two sequences, Gapped BLAST is utilized as described in Altsuchl et al. (Nucleic Acids Res. 25:3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. Homology search of proteins can be achieved, for example, on the Internet by using programs such as BLAST, FASTA, and such, for example, in databases related to amino acid sequence of polypeptides, such as SWISS-PROT, PIR, and such; databases related to polynucleotide sequences, such as DDBJ, EMBL, GenBank, and such; databases related to deduced amino acid sequences based on polynucleotide sequences;

and so on. As a result of homology search in SWISS-PROT for the amino acid sequence of SEQ ID NO:2 by using BLAST program, *Cochliobolus carbonum* tox D protein exhibited the highest percent identity (36% (Identity) and 54% positives)) among known polypeptides. Herein, a percent identity over 60% indicates, for example, the value of percent identity in Positive using BLAST program.

According to the BLAST search, potential open reading frames (ORFs), whose functions are unknown, having homology to the enone reductase of the present invention have been revealed. More specifically, three types of potential ORFs were given by genomic analysis of *Saccharomyces cerevisiae*, named YNN4, YL60, and YCZ2, respectively. Percent identity scores of these deduced amino acid sequences to the enone reductase of the present invention were 54%, 51%, and 53% (identity); and 69%, 68%, and 69% (positive), respectively. In order to clarify whether these deduced polypeptides have the enone reductase activity of the present invention, primers were synthesized based on the polynucleotide sequences deposited in the DDBJ, and the regions of potential ORFs were cloned by PCR from the genomic DNA of *Saccharomyces cerevisiae*. Each ORF was inserted in an expression vector, and *E. coli* was transformed with the vector. The resulting transformant was cultured, and each polypeptide was expressed. As a result, it was confirmed that all YNN4, YL60, and YCZ2 have the enone-reducing activity. These results confirm the validity of the presumption that a polypeptide exhibiting 60% or higher percent identity to the amino acid sequence of SEQ ID NO:2 has the enone reducing activity of the present invention. The nucleotide sequences and amino acid sequences of YNN4, YL60, and YCZ2 are shown with the following SEQ ID NOs. There is no previous report that these ORFs encode polypeptides having an enone reductase activity.

	Nucleotide sequence	Amino acid sequence
YNN4	SEQ ID NO:3	SEQ ID NO:4
YL60	SEQ ID NO:5	SEQ ID NO:6
YCZ2	SEQ ID NO:7	SEQ ID NO:8

The polynucleotides of the present invention include polynucleotides comprising the nucleotide sequences of SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7. In addition, the

present invention includes all polynucleotides comprising the nucleotide sequences encoding the amino acid sequences encoded by these polynucleotides, as well as the amino acid sequences of SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8. Further, the present invention includes polynucleotides encoding polypeptides functionally equivalent to the polypeptides consisting of the amino acid sequences of SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8.

More specifically, the polynucleotides of the present invention include polynucleotides encoding a polypeptide including any one of the amino acid sequences according to SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8, wherein one or more amino acids have been deleted, substituted, inserted and/or added, further wherein said encoded polypeptide has the enone reductase activity. Such polynucleotides can be obtained according to the method as described above.

Further, the polynucleotides of the present invention include polynucleotides hybridizing under stringent conditions to any one of the polynucleotides consisting of the nucleotide sequence of SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7, yet which encode a polypeptide having the enzyme activity of enone reductase. The "polynucleotides hybridizing under stringent conditions" refers to polynucleotides using, for example, the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech) under conditions described in the manual (wash: 42°C, primary wash buffer containing 0.5x SSC), and using polynucleotides selected from one or more sequences containing at least consecutive 20, preferably at least consecutive 30, for example, consecutive 40, 60 or 100 residues that are arbitrarily selected from the sequences of SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7 as probe polynucleotides. Also included in the invention is a polynucleotide that hybridizes under high stringency conditions to the nucleotide sequence of SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7, or a segment thereof as described herein. "High stringency conditions" refers to hybridization in 6x SSC at about 45°C, followed by one or more washes in 0.2x SSC, 0.1% SDS at 65°C.

The polynucleotides hybridizing under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 include polynucleotides which are homologous to these polynucleotides. It is highly probable that such polynucleotides encode polypeptides functionally equivalent to the

polypeptide consisting of the amino acid sequence of SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8.

Further, the polynucleotides of the present invention include polynucleotides encoding polypeptides having at least 60%, preferably at least 70% or 80%, more preferably 90% or higher percent identity to the amino acid sequence of SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8. Protein homology search can be carried out by such methods as described above.

The polynucleotides of the present invention are useful for the production of the enone reductases of the present invention by genetic engineering. With the polynucleotide of the present invention, it is also possible to create genetically engineered microorganisms having the enone reductase activity that are useful in the production of an α,β-saturated ketone from an α,β-unsaturated ketone.

The present invention includes a substantially pure polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the polypeptide has the enone reductase activity, as well as homologues thereof. The polypeptide comprising the amino acid sequence of SEQ ID NO:2 constitutes a preferred embodiment of enone reductases of the present invention.

The term "substantially pure" as used herein in reference to a given polypeptide means that the polypeptide is substantially free from other biological macromolecules. The substantially pure polypeptide is at least 75% (e.g., at least 80, 85, 95, or 99%) pure by dry weight. Purity can be measured by any appropriate standard method, for example by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

Homologues of the enone reductase of the present invention include enzymes having the amino acid sequence of SEQ ID NO:2, in which one or more amino acids are deleted, substituted, inserted and/or added. Those skilled in the art can readily obtain polynucleotides encoding such homologues of the enone reductase by properly introducing substitution, deletion, insertion, and/or addition mutations into the polynucleotide of SEQ ID NO:1 by site-directed mutagenesis (Nucleic Acid Res. 10:6487, 1982; Methods in Enzymol. 100:448, 1983; Molecular Cloning 2nd Ed., Cold Spring Harbor Laboratory Press, 1989; PCR A Practical Approach, IRL Press pp. 200, 1991), and so on.

The number of amino acids that are mutated is not particularly restricted, as long as the enone reductase activity is maintained. Normally, it is within 50 amino acids, preferably

within 30 amino acids, more preferably within 10 amino acids, and even more preferably within 3 amino acids. The site of mutation may be any site, as long as the (R)-2,3-butanediol dehydrogenase activity is maintained.

An amino acid substitution is preferably mutated into different amino acid(s) in which the properties of the amino acid side-chain are conserved. A "conservative amino acid substitution" is a replacement of one amino acid residue belonging to one of the following groups having a chemically similar side chain with another amino acid in the same group. Groups of amino acid residues having similar side chains have been defined in the art. These groups include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

Further, the homologues of the enone reductase of the present invention includes polypeptides having an amino acid sequence exhibiting at least 60%, preferably at least 70% or 80%, more preferably 90% or higher percent identity to the amino acid sequence of SEQ ID NO:2. Homology search of protein can be achieved, for example, on the Internet using programs such as BLAST, FASTA, and such, for example, in databases related to amino acid sequence of polypeptides, such as SWISS-PROT, PIR, and such; databases related to polynucleotide sequences, such as DDBJ, EMBL, GenBank, and such; databases related to deduced amino acid sequences based on polynucleotide sequences; and so on. As a result of homology search in DDBJ for the amino acid sequence of SEQ ID NO:2 by using BLAST program, Cochliobolus carbonum tox D protein exhibited the highest percent identity (36% (Identity) and 54% positives)) among known polypeptides. Herein, 60% or higher percent identity indicates, for example, the value of percent identity in Positive using BLAST program.

Potential open reading frames (ORFs), whose functions are unknown, having homology to the enone reductase of the present invention were revealed by the BLAST search. Specifically, three types of potential ORFs given by genomic analysis of *Saccharomyces cerevisiae*, which have been named YNN4, YL60, and YCZ2 were obtained.

Percent identity scores of these deduced amino acid sequences to the enone reductase of the present invention were 54%, 51%, and 53% (identity); and 69%, 68%, and 69% (positive), respectively. In order to clarify whether these candidate polypeptides have the enone reductase activity of the present invention, primers were synthesized based on the polynucleotide sequences deposited in the DDBJ, and the regions of potential ORFs were cloned by PCR from the genomic DNA of *Saccharomyces cerevisiae*. Each ORF was inserted in an expression vector, and *E. coli* was transformed with the vector. The resulting transformant was cultured, and each polypeptide was expressed. As a result, all of the polypeptides of YNN4, YL60, and YCZ2 were confirmed to have the enone-reducing activity. These results confirm the validity of presumption that a polypeptide exhibiting 60% or higher percent identity to the amino acid sequence of SEQ ID NO:2 has the enone-reducing activity of the present invention.

Namely, a polypeptide comprising any one of the amino acid sequences of SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8 constitutes a preferred embodiment of an enone reductase of the present invention.

Polynucleotides encoding an enone reductase of the present invention can be isolated, for example, by the following method.

The polynucleotides of the present invention can be isolated from other organisms by PCR cloning or hybridization based on the nucleotide sequence of SEQ ID NO:1. The nucleotide sequence of SEQ ID NO:1 is a sequence of a gene isolated from *Kluyveromyces lactis*. Polynucleotides encoding polypeptides having the enone reductase activity can be obtained from microorganisms, such as yeasts belonging to the genus *Kluyveromyces* and the genus *Saccharomyces,* by first designing PCR primers based on the nucleotide sequence of SEQ ID NO:1. For example, as described above, a polynucleotide having the nucleotide sequence of SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7 that can be isolated from *Saccharomyces cerevisiae* by PCR encodes a polypeptide having the enone reductase activity of the present invention. Alternatively, polynucleotides encoding polypeptides having a similar enzyme activity can be derived from other species using the polynucleotides, whose nucleotide sequences have already been revealed, as a probe.

Alternatively, the polynucleotides of the present invention can be obtained by utilizing the structural features of the isolated enone reductase having the physicochemical

properties described above in (A) to (C). Following the purification of the enzyme of the present invention, the N-terminal amino acid sequence is determined. Furthermore, multiple amino acid sequences can be determined by analyzing with a protein sequencer the polypeptide fragments purified by reverse-phase liquid chromatography and such, following the digestion of the purified polypeptide with enzymes, such as lysylendopeptidase and V8 protease.

Once the partial amino acid sequences are clarified, then the encoding nucleotide sequence can be estimated. PCR primers are designed based on the putative nucleotide sequence or the nucleotide sequence of SEQ ID NO:1, and then, a part of a polynucleotide of the present invention can be obtained by conducting PCR using genomic DNAs or cDNA libraries of enzyme-producing strains as the template.

Moreover, a polynucleotide of the present invention can be obtained using an obtained polynucleotide fragment as the probe, and by conducting colony hybridization, plaque hybridization, and so on, using libraries and cDNA libraries constructed by inserting the restriction enzyme digestion product of the genomic DNA of an enzyme-producing strain into a phage, plasmid, and such, and transforming *E. coli* therewith.

It is also possible to obtain a polynucleotide of the present invention by analyzing the nucleotide sequence of an obtained polynucleotide fragment by PCR, designing PCR primers to elongate the known polynucleotide, and after digesting the genomic DNA of the enzyme-producing strain with an appropriate restriction enzyme, reverse PCR is performed using the DNA as the template by a self cyclization reaction (Genetics 120, 621-623 (1988)), the RACE method (Rapid Amplification of cDNA End, "PCR experimental manual" p25-33 HBJ press), and such.

The polynucleotide of the present invention include not only genomic DNA or cDNA cloned by the above-mentioned methods but also synthesized polynucleotides.

An enone reductase-expressing vector is provided by inserting the isolated polynucleotide encoding an enone reductase of the present invention into a known expression vector. Further, by culturing cells transformed with the expression vector, the enone reductase of the present invention can be obtained from the transformed cells.

The recombinant vectors of the present invention also include recombinant vectors wherein, in addition to a polynucleotide encoding the enone reductase of the present

invention, a polynucleotide encoding a dehydrogenase catalyzing an oxidation reaction using NADP as a coenzyme is inserted. Such dehydrogenases include glucose dehydrogenase, glutamate dehydrogenase, formate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, alcohol dehydrogenase, glycerol dehydrogenase, and so on. These enzymes can be used to regenerate NADPH, the coenzyme of the enone reductases of the present invention, from $NADP^+$.

Herein, there is no restriction on the microorganism to be transformed for expressing the enone reductase, whose coenzyme is NADPH, so long as the microorganism is transformed with a recombinant vector containing a polynucleotide encoding a polypeptide having the enone reductase activity whose coenzyme is NADPH, and can express the enone reductase activity which coenzyme is NADPH. Useful microorganisms are those for which a host-vector system is available and include, for example, organisms such as:

- bacteria such as the genus *Escherichia*, the genus *Bacillus*, the genus *Pseudomonas*, the genus *Serratia*, the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Streptococcus*, and the genus *Lactobacillus*;
- actinomycetes such as the genus *Rhodococcus,* and the genus *Streptomyces*;
- yeasts such as the genus *Saccharomyces*, the genus *Kluyveromyces*, the genus *Schizosaccharomyces*, the genus *Zygosaccharomyces*, the genus *Yarrowia*, the genus *Trichosporon*, the genus *Rhodosporidium*, the genus *Pichia*, and the genus *Candida*; and
- fungi such as the genus *Neurospora*, the genus *Aspergillus*, the genus *Cephalosporium*, and the genus *Trichoderma*.

The preparation of a transformant and construction of a recombinant vector suitable for the host can be carried out by employing conventional techniques used in the fields of molecular biology, bioengineering, and genetic engineering (for example, see Sambrook et al., "Molecular Cloning", Cold Spring Harbor Laboratories). In order to express a gene encoding an enone reductase of the present invention, whose coenzyme is NADPH, in a microorganism, it is necessary to first introduce the polynucleotide into a plasmid vector or phage vector that is stable in the microorganism and allow the genetic information to transcribe and translate. Therefore, a promoter, a unit for regulating the transcription and translation, is placed upstream of the 5'-end of the polynucleotide strand of the present invention, and a terminator is preferably placed downstream of the 3'-end of the

polynucleotide strand. The promoter and the terminator should be functional in the microorganism to be utilized as the host. Available vectors, promoters, and terminators for the above-mentioned various microorganisms are described in detail in "Fundamental Course in Microbiology (8): Genetic Engineering ", Kyoritsu Shuppan, and those specifically for yeasts in "Adv. Biochem. Eng. 43, 75-102 (1990)", "Yeast 8, 423-488 (1992)", and such.

For example, for the genus *Escherichia*, in particular for *Escherichia coli*, available plasmids include the pBR series and pUC series plasmids; available promoters include promoters derived from lac (derived from β-galactosidase gene), trp (derived from the tryptophan operon), tac, trc (which are chimeras of lac and trp), P_L and P_R of λ phage, etc. Terminators derived from trpA, phages, rrnB ribosomal RNA, and so on are available. The vector pSE420D (described in the Unexamined Published Japanese Patent Application No. (JP-A) 2000-189170), which is a vector constructed by partially modifying the multicloning site of the commercially available pSE420 (Invitrogen), can be preferably used.

The pUB110 series, pC194 series plasmids, and so on can be used for the genus *Bacillus*. The vectors can be integrated into the host chromosome. Available promoters and terminators are derived from apr (alkaline protease), npr (neutral protease), amy (α-amylase), etc.

For the genus *Pseudomonas*, host-vector systems for *Pseudomonas putida* and *Pseudomonas cepacia* have been developed. A broad-host-range vector, pKT240 (containing genes required for autonomous replication derived from RSF1010, and such) based on TOL plasmid, which is involved in the decomposition of toluene compounds, is available; the promoter and terminator derived from the lipase gene (JP-A Hei 5-284973) are available.

Plasmid vectors, such as pAJ43 (Gene 39:281, 1985), are available for the genus *Brevibacterium*, in particular for *Brevibacterium lactofermentum*. Promoters and terminators used for *Escherichia coli* can be also utilized for *Brevibacterium* without any modification.

Plasmid vectors, such as pCS11 (JP-A Sho 57-183799) and pCB101 (Mol. Gen. Genet. 196:175, 1984), are available for the genus *Corynebacterium*, in particular, for *Corynebacterium glutamicum*.

Plasmid vectors, such as pHV1301 (FEMS Microbiol. Lett. 26:239, 1985) and pGK1 (Appl. Environ. Microbiol. 50:94, 1985), can be used for the genus *Streptococcus*.

Plasmid vectors, such as pAMβ1 (J. Bacteriol. 137:614, 1979), which was developed for the genus *Streptococcus*, can be utilized for the genus *Lactobacillus*; and promoters used for *Escherichia coli* can be utilized.

Plasmid vectors isolated from *Rhodococcus rhodochrous* (J. Gen. Microbiol. 138:1003, 1992) are available for the genus *Rhodococcus*.

Plasmids for the genus *Streptomyces* can be constructed according to the methods described in "Genetic Manipulation of *Streptomyces*: A Laboratory Manual" (Cold Spring Harbor Laboratories, 1985) by Hopwood et al. In particular, pIJ486 (Mol. Gen. Genet. 203:468-478, 1986), pKC1064 (Gene 103:97-99, 1991), and pUWL-KS (Gene 165:149-150, 1995) can be used for *Streptomyces lividans*. The same plasmids may be also utilized for *Streptomyces virginiae* (Actinomycetol. 11:46-53, 1997).

The YRp series, YEp series, YCp series, and YIp series plasmids are available for the genus *Saccharomyces*, in particular, for *Saccharomyces cerevisiae*. Integration vectors (refer EP 537456, etc.) that utilize the homologous recombination with the ribosomal DNA, many copies of which exist on the chromosome, allow introduction of genes of interest in multicopy and those genes incorporated are stably maintained in the microorganism; thus, these types of vectors are highly useful. Promoters and terminators derived from genes encoding ADH (alcohol dehydrogenase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PHO (acid phosphatase), GAL (β-galactosidase), PGK (phosphoglycerate kinase), ENO (enolase), and so on can be utilized.

For the genus *Kluyveromyces*, in particular, for *Kluyveromyces lactis*, plasmids such as 2-μm series plasmids derived from *Saccharomyces cerevisiae*; the pKD1 series plasmids (J. Bacteriol. 145:382-390, 1981); plasmids derived from pGKl1 involved in the killer activity, KARS (*Kluyveromyces* autonomous replication sequence) series plasmids; and vector plasmids (refer EP 537456, etc.), which can be integrated into the chromosome through the homologous recombination with the ribosomal DNA and such, are available. Promoters and terminators derived from ADH, PGK, and the like are available.

Plasmid vectors comprising the ARS (autonomous replication sequence) derived from *Schizosaccharomyces pombe* and the auxotrophy-complementing selectable markers derived from *Saccharomyces cerevisiae* (Mol. Cell. Biol. 6:80, 1986) are available for the genus *Schizosaccharomyces*. Promoters such as ADH promoter derived from *Schizosaccharomyces*

pombe may be used (EMBO J. 6:729, 1987). In particular, pAUR224 is commercially available from TaKaRa Shuzo Co., Ltd., and thus, can be used readily.

Plasmid vectors originating from pSB3 (Nucleic Acids Res. 13:4267, 1985); derived from *Zygosaccharomyces rouxii*), and such are available for the genus *Zygosaccharomyces*. Promoters such as PHO5 promoter derived from *Saccharomyces cerevisiae* and GAP-Zr (Glyceraldehyde-3-phosphate dehydrogenase) promoter (Agri. Biol. Chem. 54:2521, 1990) derived from *Zygosaccharomyces rouxii* can be used.

Host vector systems utilizing *Pichia*-derived genes involved in autonomous replication (PARS1 and PARS2) are developed for the genus *Pichia*, especially for *Pichia pastoris* and such (Mol. Cell. Biol. 5:3376, 1985), and thus, strong promoters such as AOX, which can be cultivated to high-density and are inducible with methanol (Nucleic Acids Res. 15:3859, 1987) are available. Additionally, another host-vector system has been developed for *Pichia angusta* (previously called *Hansenula polymorpha*) among the genus *Pichia*. Vectors including *Pichia angusta*-derived genes (HARS1 and HARS2) involved in autonomous replication are also useful; however, they are relatively unstable. Therefore, multi-copy integration of the gene into the chromosome is effective (Yeast 7:431-443, 1991). Promoters of AOX (alcohol oxidase) and FDH (formic acid dehydrogenase), which are induced by methanol and such, are also available.

For the genus *Candida*, host-vector systems have been developed for *Candida maltosa*, *Candida albicans*, *Candida tropicalis*, *Candida utilis*, etc. An autonomous replication sequence (ARS) originating from *Candida maltosa* has been cloned (Agri. Biol. Chem. 51:1587, 1987), and a vector using the sequence has been developed for *Candida maltosa*. Further, a chromosome-integration vector with a highly efficient promoter unit has been developed for *Candida utilis* (JP-A Hei. 08-173170).

In relation to the genus *Aspergillus*, *Aspergillus niger* and *Aspergillus oryzae* have been intensively studied among fungi, and thus, both plasmid vectors and chromosome-integration vectors are available. Furthermore, promoters derived from an extracellular protease gene and amylase gene (Trends in Biotechnology 7:283-287, 1989) are available.

Host-vector systems have been developed for *Trichoderma reesei* of the genus *Trichoderma*, and promoters such as those derived from an extracellular cellulase gene, and such are available (Biotechnology 7:596-603, 1989).

There are various host-vector systems developed for plants and animals other than microorganisms; in particular, the systems include those of insect, such as silkworm (Nature 315:592-594, 1985), and plants, such as rapeseed, maize, potato, etc. These systems are preferably employed to express a large amount of foreign polypeptides.

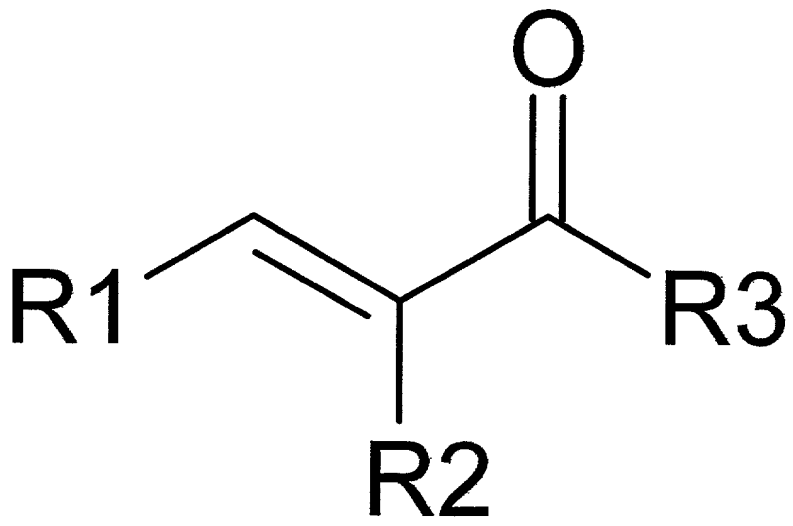
Further, transformants expressing an enone reductase of the present invention obtained by the above methods can be used to produce an enzyme of the present invention, as well as to produce α,β-saturated ketone by selective reduction of the carbon-carbon double bond of α,β-unsaturated ketone as described below.

Namely, the present invention relates to methods for selectively reducing the carbon-carbon double bond of α,β-unsaturated ketone, which comprises the step of reacting the α,β-unsaturated ketone with any one of the materials exhibiting the enzymatic activity selected from the group consisting of the above-mentioned enone reductases, microorganisms producing the enzymes or polypeptides, and processed products of the microorganisms. The desired enzyme reaction can be carried out by contacting the reaction solution with an enzyme of the present invention, a culture containing an enzyme, or processed products thereof.

According to the method of the present invention, polypeptides comprising the amino acid sequence of SEQ ID NO:2, homologues thereof, and enone reductases having the above physicochemical properties (A) to (C) can be used as enone reductases. Not only purified enone reductases but also crude enzymes are usable. Further, cells producing the enone reductase can be also used as an enone reductase according to the present invention. All strains belonging to the genus *Kluyveromyces*, mutant strains, variants, and genetically engineered transformants that have acquired the productivity of enzyme of the present invention, which can produce the NADPH-dependent enone reductase are included as enone reductase producing cells to be used in the present invention. The enone reductase producing cells can be used in the form of the culture, cells separated from the culture medium by filtration, centrifugation or the like, or cells resuspended in buffer, water, or the like after they are separated by centrifugation and washed. The separated cells can be used in a state as they are recovered, as their disrupts, as treated with acetone or toluene, or as lyophilizate. When the enzyme is extracellularly produced, the culture medium of the cells can also be used after it is separated from the cells by the usual methods.

The means by which the enzymes and the reaction solutions are contacted is not limited to these specific examples. The reaction solution comprises substrates and NADPH, a coenzyme required for the enzyme reaction, dissolved in a suitable solvent that gives an environment desirable for enzyme activity. Specific examples of processed products of microorganisms containing an enone reductase of the present invention include: microorganisms, wherein the permeability of the cell membrane has been altered by detergents or organic solvents, such as toluene; cell-free extracts obtained by lysing the microorganism with glass beads or by enzyme treatment; partially purified material of the cell-free extracts; and so on.

There is no limitation on the α,β-unsaturated ketones of the present invention. For example, the α,β-unsaturated ketones include compounds represented by the following formula I;



wherein:

R1 is a substituted or unsubstituted alkyl group, a substituted or unsubstituted alkenyl group, a substituted or unsubstituted aralkyl group, or a substituted or unsubstituted alkoxy group;

R2 is hydrogen, or a substituted or unsubstituted short-chain alkyl group; and

R3 is a substituted or unsubstituted short-chain alkyl group.

More specifically, methyl vinyl ketone, ethyl vinyl ketone, 3-penten-2-one, 3-methyl-3-penten-2-one, and such are suitably used.

Further, the enzyme can also be utilized for the synthesis of optically active saturated ketones by allowing the enzymes of the present invention, microorganisms producing the enzyme, or processed products thereof to react on α,β-unsaturated ketones containing α-substitution.

An NADPH regeneration system can be combined with the method for producing ketones according to the above-mentioned present invention. The reduction by enone reductases accompanies generation of $NADP^+$ from NADPH. Regeneration of NADPH from $NADP^+$ can be achieved by using enzymes (systems) regenerating NADPH from $NADP^+$ contained in microorganisms. It is possible to enhance the ability of the enzymes (systems) to reduce $NAPD^+$ by adding glucose or ethanol into the reaction system. Furthermore, NADPH can be regenerated using microorganisms including enzymes which have the ability to generate NADPH from $NAPD^+$, for example, glucose dehydrogenase, glutamate dehydrogenase, formic acid dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, alcohol dehydrogenase, glycerol dehydrogenase, and so on; and processed products thereof; as well as partly purified and purified enzymes. For example, the regeneration of NADPH can be achieved by using the conversion of glucose to δ-gluconolactone catalyzed by the above glucose dehydrogenase.

The components required for the reaction to regenerate NADPH can be added or added after immobilization on a solid phase to the reaction system to produce ketones in accordance with the present invention. Alternatively, they can be contacted *via* a membrane which permeates NADH.

Furthermore, in some cases where living microorganism transformed with recombinant vectors containing the polynucleotide of the present invention are used in the production of ketones described above, additional reaction systems for the regeneration of NADPH are unnecessary. Specifically, efficient reaction can be achieved without the addition of enzymes for the regeneration of NADPH by using microorganisms that have a higher activity for regenerating NADPH in the reduction reaction with transformants. Furthermore, it is possible to conduct a more efficient reaction to express the NADPH regenerating enzymes and NADPH-dependent enone reductases, and to conduct a more

efficient reduction reaction by co-introducing a gene encoding glucose dehydrogenase, formic acid dehydrogenase, alcohol dehydrogenase, amino acid dehydrogenase, organic acid dehydrogenase (e.g., malate dehydrogenase), or the like, which can be utilized in the regeneration of NADPH, together with a polynucleotide encoding the NADPH-dependent enone reductase of the present invention into a host. Several methods are available for introducing these two genes or more into a host, including methods to transform a host with multiple recombinant vectors derived from different origins separately inserted with each gene to avoid incompatibility in *E. coli*; methods wherein both genes are inserted into a single vector; methods wherein either or both genes are introduced into the chromosome; and so on.

Bacillus subtilis-derived glucose dehydrogenase can be mentioned as a glucose dehydrogenases that can be used to regenerate NADPH in the present invention. The gene encoding the enzyme has been already isolated. Based on the known nucleotide sequence, the gene can also be obtained from the microorganism by PCR or hybridization screening.

When multiple genes are intended to be inserted into a single vector, they can be expressed by methods wherein the control regions associated with expression, such as promoter and terminator, are ligated with each gene and by methods wherein the genes are expressed as operons containing multiple cistrons, such as lactose operon.

The reduction reaction using an enzyme of the present invention may be performed in water or in a two-solvent system consisting of water and organic solvent that is not miscible with water. For example, ethyl acetate, butyl acetate, toluene, chloroform, n-hexane, isooctane, and such are included as usable organic solvents that is not miscible with water. Alternatively, the reaction can be also carried out in a mixed solvent system consisting of aqueous solvent and organic solvent such as ethanol, acetone, dimethyl sulfoxide, acetonitrile, etc.

The reaction of the present invention can be also conducted by using immobilized enzymes, membrane reactors, and so on. α,β-unsaturated ketones used as substrates in the reaction are often insoluble in water. Therefore, the inhibitory effects of the substrate and product can be reduced by contacting and reacting the aqueous phase containing the enzyme of the present invention, microorganism containing the enzyme of the present invention, or processed products thereof with the organic solvent phase containing the substrate,

α,β-unsaturated ketone, through a hydrophobic membrane, such as polypropylene membrane.

The enzyme reaction of the enone reductase of the present invention can be carried out under the following condition:

reaction temperature: 4 to 55°C, preferably 10 to 45°C;

pH: 4 to 9, preferably 5.5 to 8, more preferably pH6.5 to 7.0; and

substrate concentration: 0.01 to 90%, preferably 0.1 to 20%.

The coenzyme $NADP^+$ or NADPH can be added at a concentration of 0.001 mM to 100 mM, preferably 0.01 to 10 mM, to the reaction system, according to needs. The substrate can be added once at the start of reaction, but it is preferably added continuously or stepwise to prevent the substrate concentration in the reaction solution from becoming too high.

Compounds added to the reaction system to regenerate NADPH (e.g., glucose when glucose dehydrogenase is used, formic acid when formate dehydrogenase is used, ethanol or 2-propanol when alcohol dehydrogenase is used, L-glutamic acid when glutamate dehydrogenase is used, and L-malic acid when malate dehydrogenase is used, etc.) can be added at a molar ratio of 0.1-20, preferably 0.5-5 to the substrate, α,β-unsaturated ketone. The enzymes for regenerating NADPH, for example, glucose dehydrogenase, formate dehydrogenase, alcohol dehydrogenase, amino acid dehydrogenase, organic acid dehydrogenase (malate dehydrogenase, etc.), and such, can be added at an enzymatic activity of 0.1-100 folds, preferably 0.5-20 folds as compared to the enzymatic activity of the NADPH-dependent enone reductase of the present invention.

The purification of ketone generated by the reduction of α,β-unsaturated ketone according to the present invention can be performed by properly combining centrifugation of fungal cells and polypeptides, separation with membrane and such, extraction by solvent, distillation, chromatography, and so on.

The enzymes of the present invention to be used in various synthetic reactions and are not restricted to purified enzymes. They also include partially purified enzymes, cells of microorganisms containing the enzyme, processed products thereof, and so on. The processed product of the present invention includes cells of microorganisms, purified enzymes, partially purified enzymes, and such, that are immobilized by various methods.

The immobilization can be achieved by a known method such as sulfur-containing polysaccharide (e.g., κ-carrageenan), calcium alginate, agar gel method, and polyacrylamide gel method.

Novel enone reductases that selectively reduce the carbon-carbon double bond of α,β-unsaturated ketone are provided. Ketones useful as a raw material for pharmaceuticals can be enzymatically produced using such enzymes. The enone reductases of the present invention have high selectivity toward the carbon-carbon double bond of α,β-unsaturated ketone. Therefore, the ketone of interest can be prepared at a high yield.

Any patents, patent applications, and publications cited herein are incorporated by reference.

The present invention is illustrated in detail below with reference to Examples, but is not to be construed as being limited thereto.

Herein, "%" for concentration denotes weight per volume percent unless otherwise specified.

Example 1. Purification of enone reductase

Kluyveromyces lactis, IFO 1267 strain, was cultured in 1.2 L of YM culture medium (20 g/L glucose, 3 g/L yeast extract, 3 g/L wheat germ extract, 5 g/L peptone; pH 6.0). The fungal cells were harvested by centrifugation. The resulting wet fungal cells were suspended in a solution consisting of 50 mM potassium phosphate buffer (pH 8.0), 0.02% 2-mercaptoethanol, and 2 mM phenyl methane sulfonylfluoride (PMSF), and then, crushed with a bead-beater (Biospec). Then, fungal-cell debris was removed by centrifugation and the cell-free extract was obtained. Nucleic acid-free supernatant was prepared by adding protamine sulfate to the cell-free extract followed by centrifugation of the mixture. Ammonium sulfate was added to the supernatant to 30% saturation. The supernatant was loaded onto a column of phenyl-Sepharose HP (2.6 cm x 10 cm) equilibrated with a standard buffer (10 mM Tris-HCl buffer (pH 8.5), 0.01% 2-mercaptoethanol, 10% glycerol) containing 30% ammonium sulfate; the elution was performed with a concentration gradient of 30 to 0% ammonium sulfate.

The NADPH-dependent methyl vinyl ketone reducing activity was eluted as two peaks by the elution with the concentration gradient. Eluted fractions corresponding to the second peak of the two were collected, and were concentrated by ultrafiltration.

The concentrated enzyme solution was dialyzed against the standard buffer, and then, was loaded onto the MonoQ (0.5 cm x 5 cm) equilibrated with the same buffer. After the column was washed with the standard buffer, elution was carried out with a concentration gradient of 0-0.5 M sodium chloride. The eluted active fractions were collected, and were concentrated by ultrafiltration.

Ammonium sulfate was added to the concentrated enzyme solution at 30% saturation. The solution was loaded onto the phenyl-Superose (0.5cmx5cm) equilibrated with the standard buffer containing 30% saturated ammonium sulfate. After the column was washed with the same buffer, elution with a gradient of saturated ammonium sulfate of 30-0% was carried out. The eluted active fractions were collected.

The active fractions obtained by using the phenyl-Superose were analyzed by SDS-PAGE; the fraction gave a single band (FIG. 1).

Specific activity of the purified enzyme was about 31.7 U/mg. The purification processes are summarized in Table 1.

Table 1

Step	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg)
Cell-free extract	3390	1360	0.401
Protamine sulfate precipitation	1480	1220	0.851
Phenyl-Sepharose	156	222	1.42
MonoQ	2.70	117	43.4
Phenyl-Superose	0.162	5.14	31.7

Example 2. Molecular weight determination of enone reductase

The molecular weight of the subunit of the enzyme obtained in Example 1 was determined to be 43,000 by SDS-PAGE. Further, the molecular weight determined by using a gel filtration column, Superdex G200, was approximately 42,000. Therefore, the enone reductase of the present invention was predicted to be a monomer.

Example 3. Optimal pH of enone reductase

The methyl vinyl ketone-reducing activity of the enzyme obtained in Example 1 was tested, by altering the pH of the reaction with potassium phosphate buffer and Britton Robinson buffer. The activity is represented by a relative activity, taking the maximal activity as 100, and the results are shown in FIG. 2. The optimal pH for the reaction was determined to be 6.5 to 7.0.

Example 4. Optimal temperature for enone reductase

The methyl vinyl ketone-reducing activity of the enzyme obtained in Example 1 was assayed under standard reaction conditions, with the exception that only the temperatures were altered. The activity is represented by a relative activity, taking the maximal activity as 100, and the results are shown in FIG. 3. The optimal temperature was 37 to 45°C.

Example 5. Substrate specificity of enone reductase

The enzyme obtained in Example 1 was reacted with various enones, ketones, and aldehydes, and the dehydrogenation activity was assayed. The result was represented by a relative activity, taking the dehydrogenation activity of the enzyme on methyl vinyl ketone as 100, and the results are shown in Table 2.

Table 2

Substrate	coenzyme	Relative activity (%)
Methyl vinyl ketone	NADPH	100
Ethyl vinyl ketone	NADPH	537
3-pentene-2-one	NADPH	16

4-methyl-3-pentene-2-one	NADPH	1
3-methyl-3-pentene-2-one	NADPH	48
2-methyl-2-cyclopenten-1-one	NADPH	0
3-methyl-2-cyclopenten-1-one	NADPH	0
2-butanone	NADPH	0
Crotonic acid	NADPH	0
Methylglyoxal	NADPH	1
2,3-butanedione	NADPH	1
Acetophenone	NADPH	0
Methyl vinyl ketone	NADH	14
Ethyl vinyl ketone	NADH	52

Example 6. Synthesis of 3-pentanone using enone reductase

The reaction was carried out overnight in a reaction solution containing 200 mM potassium phosphate buffer (pH 6.5), 44 mg NADH, 1 U enone reductase, and 0.2% ethyl vinyl ketone at 25°C. The produced 3-pentanone was quantified by gas chromatography, and the yield was determined based on the quantity of the starting material, ethyl vinyl ketone. The condition used for gas chromatography was as follows: Porapak PS (Waters, mesh 50-80, 3.2 mm x 210 cm) was used; the column temperature was 130°C; the analysis was carried out with a flame ionization detector (FID). The result showed that the reaction yield was 100%.

Example 7. Partial amino acid sequence of enone reductase

The enzyme obtained in Example 1 was fractionated by SDS-PAGE; a gel piece containing the enone reductase was cut out. After washing the gel piece twice, the enzyme was digested overnight in the gel with lysylendopeptidase at 35°C. The digested peptide was fractionated and obtained using reverse HPLC (TSK gel ODS-80-Ts, 2.0 mm x 250 mm; Tosoh) by the elution with an acetonitrile gradient in 0.1% trifluoroacetic acid (TFA).

The obtained two peaks of peptide fractions were named lep_64 and lep_65, respectively. Each fraction was analyzed for the amino acid sequence in a protein sequencer

(Hewlett Packard G1005A Protein Sequencer System). The amino acid sequences for lep_64 and lep_65 are shown in SEQ ID NOs: 9 and 10, respectively.

SEQ ID NO:9:lep_64

Ser-Tyr-Gly-Ala-Asp-Asp-Val-Phe-Asp-Tyr-His-Asp

SEQ ID NO:1 0:lep_65

Ile-Gly-Pro-Glu-Gly-Ser-Ile-Leu-Gly-Cys-Asp-Ile

Example 8. Purification of chromosomal DNA from *Kluyveromyces lactis*

Kluyveromyces lactis, IFO 1267 strain, was cultured in YM culture medium, and the fungal cells were prepared. The purification of chromosomal DNA from the fungal cells was carried out by the method as described in "Meth. Cell Biol. 29, 39-44 (1975)".

Example 9. Cloning of the core region of enone reductase gene

Three kinds of sense and antisense primers in total were synthesized based on the amino acid sequences of lep_64 and lep_65. Respective nucleotide sequences are shown in SEQ ID NO:11 (KR2-64U), 12 (KR2-65D), and 13 (KR2-65E).

SEQ ID NO:11: KR2-64U

TGRTARTCRAANACRTCRTC

SEQ ID NO:12: KR2-65D

ATWGGHCCWGARGGHTCNAT

SEQ ID NO:13: KR2-65E

ATWGGHCCNGARGGHAGYAT

Two of the three primers were selected as a combination. PCR amplification was conducted with 50 μL reaction solution containing: primers (50 pmol each), 10 nmol dNTP, 50 ng chromosomal DNA derived from *Kluyveromyces lactis*, AmpliTaq buffer (Takara Shuzo), and 2 U AmpliTaq (Takara Shuzo); 30 cycles of denaturation (at 94°C for 30 seconds), annealing (at 45°C for 30 seconds), and extension (at 70°C for 1 minute) on a GeneAmp PCR System 2400 (Perkin Elmer) was carried out.

An aliquot of the PCR reaction solution was analyzed by agarose gel electrophoresis; a band, which was assumed to be specific, was detected from solutions containing KR2-64U and KR2-65D as primers. The obtained DNA fragment was extracted with

phenol/chloroform, precipitated by ethanol, and the precipitate was collected. The obtained DNA fragment was ligated to pT7Blue (R) T-vector (Novagen) using the Takara Ligation Kit, and then was transformed into *E. coli* JM109 strain.

The transformed strain was grown on a plate of LB culture medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% sodium chloride; hereinafter abbreviated as LB culture medium) containing ampicillin (50 μg/mL); several white colonies were selected by the blue/white selection method. The length of the inserts in the selected white colonies were checked by colony-direct PCR using commercially available primers M13-21 (TGTAAAACGACGGCCAGT (SEQ ID NO:28)) and M13-RP (CAGGAAACAGCTATGACC (SEQ ID NO:29)). The colonies, which were presumed to contain the DNA fragment of interest as an insert, were cultured in LB liquid culture medium containing ampicillin. The plasmid was purified with Flexi-Prep (Pharmacia), and was named pKLR2.

The nucleotide sequence of the DNA insert was analyzed using the purified plasmid. Nucleotide sequence analysis of the DNA was carried out with a DNA sequencer ABI PRISM™ 310 (Perkin Elmer), after the DNA was amplified by PCR using the BigDye Terminator Cycle Sequencing FS ready Reaction Kit (Perkin Elmer). The determined nucleotide sequence of the core region is shown in SEQ ID NO:14.

Example 10. Nucleotide sequence analysis of DNA regions adjacent to the core region of the enone reductase gene

Chromosomal DNA derived from *Kluyveromyces lactis* was digested with the restriction enzyme, *Hae*II or *Pst*I, and then, was self-ligated overnight at 16°C using T4 ligase to cyclize each fragment. Then, PCR amplification was conducted in a 50 μL reaction solution containing: primers KL2-5U (SEQ ID NO:15) and KL2-3D (SEQ ID NO:16) (100 pmol each); 25 ng circular DNA; Ex-Taq buffer (Takara Shuzo); and 2 U Ex-Taq (Takara Shuzo). 30 cycles of denaturation (at 94°C for 30 seconds), annealing (at 55°C for 30 seconds), and extension (at 72°C for 7 minutes) on a GeneAmp PCR System 2400 (Perkin Elmer) was carried out. An aliquot of the PCR reaction solution was analyzed by agarose gel electrophoresis; a band of approximately 5000 bp, which was assumed to be specific, was

detected. The DNA fragment was purified with a Sephaglas BandPrep Kit (Pharmacia). The nucleotide sequence of the fragment was determined by the primer walking method.

Five kinds of primers were used: KL2-5U, KL2-3D, KL2-Sq1 (SEQ ID NO:17), KL2-Sq2 (SEQ ID NO:18), and KL2-Sq3 (SEQ ID NO:19). Nucleotide sequence analysis of the DNA was carried out by a DNA sequencer ABI PRISM™ 310 (Perkin Elmer), after the DNA was amplified by PCR using the BigDye Terminator Cycle Sequencing FS ready Reaction Kit (Perkin Elmer). Thus, the ORF sequence of enone reductase was determined. The determined DNA sequence is shown in SEQ ID NO:1; and the sequence of the encoded protein is shown in SEQ ID NO:2. ORF search from the DNA sequence, translation from the ORF to the deduced amino acid sequence, and others, were performed with Genetyx-WIN (Software Development Co., LTD).

SEQ ID NO:15: KL2-5U

TCCGGTACCGACAACTGTACCAGCAATGTC

SEQ ID NO:16: KL2-3D

ATCGGTACCTATACTAAGATTGTAACTGTTGC

SEQ ID NO:17: KL2-Sq1

CCGGGTACCCTTTTAGGGTGA

SEQ ID NO:18: KL2-Sq2

TCATGAAGCCACAGTTAAATTCG

SEQ ID NO:19: KL2-Sq3

ATATTCATATGATGGATATCACCG

Example 11. Cloning of the enone reductase gene

Primers for ORF cloning were synthesized based on the sequence of the structural gene of the enone reductase: KLCR2-N (SEQ ID NO:20), and KLCR2-C (SEQ ID NO:21). PCR amplification was conducted in 50 μL reaction solution containing: primers (50 pmol each); 10 nmol dNTP; 50 ng chromosomal DNA derived from *Kluyveromyces lactis*; Pfu Turbo-DNA polymerase buffer (STRATAGENE); and 2.5 U Pfu Turbo-DNA polymerase (STRATAGENE). 30 cycles of denaturation (at 95°C for 150 seconds), annealing (at 55°C for 1 minute), and extension (at 75°C for 90 seconds) on a GeneAmp PCR System 2400 (Perkin Elmer) was carried out.

SEQ ID NO:20: KLCR2-N

CTGGAATTCTACCATGGCTTCAGTTCCAACCACTCAAAAAG

SEQ ID NO:21: KLCR2-C

GACAAGCTTCTAGATTATAACCTGGCAACATACTTAACA

An aliquot of the PCR reaction solution was analyzed by agarose gel electrophoresis; a band, which was assumed to be specific, was detected.

The obtained DNA fragment was extracted with phenol/chloroform, precipitated with ethanol, and then, was collected. The DNA fragment was double-digested with restriction enzymes, *Nco*I and *Xba*I, and then, was electrophoresed on an agarose gel. The band of interest was cut out, and the DNA was purified with the Sepaglas BandPrep Kit (Pharmacia). The obtained DNA fragment was ligated to pSE420D, which had been double-digested with *Nco*I and *Xba*I, using the Takara Ligation Kit. The ligate was transformed into *E. coli* JM109 strain.

The transformed strain was grown on a plate of LB culture medium containing ampicillin (50 μg/mL); and the length of inserts in several colonies were checked by colony-direct PCR using KLCR2-N and KLCR2-C primers. Plasmids were purified from colonies confirmed to contain inserts of the desired size. Then, the nucleotide sequence of the insert fragment was analyzed. The plasmid containing the object enone reductase gene was designated as pSE-KLR1 (FIG. 4).

Example 12. Production of recombinant enone reductase in *E. coli*

E. coli HB101 strain, transformed with plasmid pSE-KLR1 expressing the enone reductase, was cultured overnight in liquid LB medium containing ampicillin at 30°C. 0.1mM IPTG was added to the culture, and then, was further cultured for 4 hours.

The bacterial cells were harvested by centrifugation, and then, were suspended in 50mM potassium phosphate buffer (pH8.0) containing 0.02% 2-mercaptoethanol, 2mM PMSF, and 10% glycerin. The cells were treated in a closed-chamber sonicator UCD-200TM (Cosmo Bio) for 3 minutes to crush the cells. The bacterial cell lysate was centrifuged and the supernatant was recovered as bacterial cell extract. The extract was assayed for activities to various types of substrates. In addition, *E. coli* HB101 strain without the plasmid was cultured overnight in LB culture medium. 0.1 mM IPTG was added to the

culture, and then, was further cultured for 4 hours. The bacterial cells were crushed by the same method as above, and the extract was assayed for the activities to various types of substrates. These results are shown in Table 3.

Table 3

Substrate	Host only	HB101 (pSE-KLR1)	
	Specific activity (U/mg)	Specific activity (U/mg)	Relative activity (%)
Methyl vinyl ketone	0.066	7.78	100
Ethyl vinyl ketone	0.073	41.8	537
3-pentene-2-one	0.015	1.23	15.9
3-methyl-3-pentene-2-one	0.004	2.52	32.4

Example 13. Purification of chromosomal DNA from *Saccharomyces cerevisiae*

Saccharomyces cerevisiae X2180-1B (Yeast Genetic Stock Center) was cultured in YM culture medium, and the fungal cells were harvested. The purification of chromosomal DNA from the fungal cells was carried out by the method described in "Meth. Cell Biol. 29, 39-44 (1975)".

Example 14. Cloning of enone reductase homologue, YNN4

PCR primers, YNN4-ATG1 (SEQ ID NO:22) and YNN-TAA1 (SEQ ID NO:23), were synthesized based on the DNA sequence (DDBJ Accession No. Z46843) corresponding to a putative protein YNN4 (SWISS-PROT Accession No., P53912) deposited in DDBJ.

PCR amplification was conducted in 50 μL reaction solution containing: primers (25 pmol each); 10 nmol dNTP, 50 ng chromosomal DNA derived from *Saccharomyces cerevisiae*; Pfu DNA polymerase buffer (STRATAGENE); and 2 U Pfu DNA polymerase (STRATAGENE). 30 cycles of denaturation (at 95°C for 45 seconds), annealing (at 50°C for 1 minute), and extension (at 75°C for 6 minutes) on a GeneAmp PCR System 2400 (Perkin Elmer) was carried out. Specific amplification products were provided.

The amplification products were treated with phenol, and then, were double-digested with restriction enzymes, *Afl*III and *Xba*I. The resulting fragment was ligated with vector pSE420D, which had been double-digested with restriction enzymes *Nco*I and *Xba*I, using the TAKARA Ligation Kit. *E. coli* JM109 strain was transformed with the ligated DNA, and then, was grown on a plate of LB culture medium containing ampicillin (50 mg/L). Plasmids were purified from the resulting transformant with FlexiPrep. The obtained plasmid was designated as pSE-YNN4.

The nucleotide sequence of the insert DNA in the plasmid was analyzed. The revealed sequence is shown in SEQ ID NO:3. The determined nucleotide sequence perfectly agreed with the nucleotide sequence deposited in DDBJ. The amino acid sequence deduced from the nucleotide sequence of SEQ ID NO:3 is shown in SEQ ID NO:4.

SEQ ID NO:22: YNN4-ATG1

CAAACATGTCTGCCTCGATTCCAGA

SEQ ID NO:23: YNN4-TAA1

CAGTCTAGATTATTTCAAGACGGCAACCAAC

Example 15. Cloning of enone reductase homologue, YL60

PCR primers, YL60-ATG2 (SEQ ID NO:24) and YL60-TAA1 (SEQ ID NO:25), were synthesized based on the DNA sequence (DDBJ Accession No. U22383) corresponding to a putative protein YL60 deposited in DDBJ (SWISS-PROT Accession No. P54007).

PCR amplification was conducted in 50 μL reaction solution containing: primers (25 pmol each); 10 nmol dNTP; 50 ng chromosomal DNA derived from *Saccharomyces cerevisiae*; Pfu DNA polymerase buffer (STRATAGENE); and 2 U Pfu DNA polymerase (STRATAGENE). 30 cycles of denaturation (at 95°C for 45 seconds), annealing (at 50°C for 1 minute), and extension (at 75°C for 6 minutes) on a GeneAmp PCR System 2400 (Perkin Elmer) was carried out. Specific amplification products were provided.

The amplification products were treated with phenol, and then, were double-digested with restriction enzymes, *Nco*I and *Xba*I. The resulting fragment was ligated with vector pSE420D, which had been double-digested with restriction enzymes *Nco*I and *Xba*I, using the TAKARA Ligation Kit.

E. coli JM109 strain was transformed with the ligated DNA, and then, was grown on a plate of LB culture medium containing ampicillin (50 mg/L). The plasmid was purified from the resulting transformant with FlexiPrep. The obtained plasmid was designated as pSE-YL60.

The nucleotide sequence of the insert DNA in the plasmid was analyzed. The revealed sequence is shown in SEQ ID NO:5. The determined nucleotide sequence perfectly agreed with the nucleotide sequence deposited in DDBJ. The amino acid sequence deduced from the nucleotide sequence of SEQ ID NO: 5 is shown in SEQ ID NO:6.

SEQ ID NO:24: YL60-ATG2

CAACCATGGCTCAAGTTGCAATTCCAGAAACC

SEQ ID NO:25: YL60-TAA1

GACTCTAGATTAGTTTAATACGGCAACGAGTTTTTCAC

Example 16. Cloning of enone reductase homologue, YCZ2

PCR primers, YCZ2-ATG1 (SEQ ID NO:26) and YCZ2- TAA1 (SEQ ID NO:27), were synthesized based on the DNA sequence (DDBJ Accession No. X59720) corresponding to a putative protein YCZ2 deposited in DDBJ (SWISS-PROT Accession No., P25608).

PCR amplification was conducted in 50 μL reaction solution containing: primers (25 pmol each); 10 nmol dNTP; 50 ng chromosomal DNA derived from *Saccharomyces cerevisiae*; Pfu DNA polymerase buffer (STRATAGENE); and 2U Pfu DNA polymerase (STRATAGENE). 30 cycles of denaturation (at 95°C for 45 seconds), annealing (at 50°C for 1 minute), and extension (at 75°C for 6 minutes) on a GeneAmp PCR System 2400 (Perkin Elmer) was carried out. Specific amplification products were provided.

The amplification products were treated with phenol, and then, were double-digested with restriction enzymes, *Bsp*HI and *Xba*I. The resulting fragment was ligated with vector pSE420D, which had been double-digested with restriction enzymes *Nco*I and *Xba*I using the TAKARA Ligation Kit.

E. coli JM109 strain was transformed with the ligated DNA, and then, was grown on a plate of LB culture medium containing ampicillin (50 mg/L). The plasmid was purified from the resulting transformant with FlexiPrep. The plasmid obtained was designated as pSE-YCZ2.

The nucleotide sequence of the insert DNA in the plasmid was analyzed. The revealed sequence is shown in SEQ ID NO:7. While the nucleotide "C" had been substituted for "A" at nucleotide residue 1089 in the determined nucleotide sequence, the sequence of the encoded amino acid was the same as that deposited in the databank. The amino acid sequence deduced from the nucleotide sequence is shown in SEQ ID NO:8.

SEQ ID NO:26: YCZ2-ATG1

GAAATCATGAAAGCTGTCGTCATTGAA

SEQ ID NO:27: YCZ2-TAA1

GTTTCTAGATTAGTTTAATACGGCAACKAGTTTTTCA

Example 17. Verification of the activity of the enone reductase homologues, YNN4, YL60, and YCZ2

E. coli JM109 strains, each containing pSE-YNN4, pSE-YL60 or pSE-YCZ2, were cultured in LB culture medium containing ampicillin. The induction of the enzyme was achieved by adding 0.1 mM IPTG and culturing for 4 hours. The bacterial cells were harvested by centrifugation. Respective bacterial cells were suspended in cell lysis buffer (50 mM KPB (pH 8.0), 1 mM EDTA, 0.02% 2-ME, 2 mM PMSF, and 10% glycerol); and the cells were lysed in a sonicator. The supernatant prepared by centrifugation was used as the cell-free extract.

Each of the cell-free extract was assayed for the enone reductase activity. The cell-free extracts exhibited activities of 0.268 U/mg protein, 0.198 U/mg protein and 0.133 U/mg protein, respectively. Thus, it was verified that all of the three types of homologues of the enzyme of the present invention had the requisite enone reductase activity.